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Parathyrin Assay
An Analytical Evaluation of two Commercial Immuno Radiometric Assay Kits

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Summary: We have performed a comparative evaluation of two Immuno Radiometric Assay (IRMA) kits for parathyrin against an existing Radioimmuno Assay (RIA) technique for the measurement of intact parathyrin.

The analytical evaluation which was performed in line with the ECCLS recommended kit evaluation protocol showed a marginal improvement in precision with the new assays. There was a substantial improvement in the theoretical limit of detection utilising the IRMA kits although it may prove difficult to realize this improvement in practice with individual samples because of differing protein matrices. The evaluation also demonstrated the degree of parallelism and range of linearity of both kits as well as inaccuracies when compared with the International Reference Preparation for parathyrin (IRP 79/500) as the accepted standard.

The demonstration of lack of agreement between measured and kit assigned results for standards when cross-over studies between kits were performed may highlight a possible contributing factor to inaccuracy. Alternatively there may be a difference of antisera avidity within the kits for intact parathyrin.

Whilst minor differences in sample stability were demonstrated between the kits, the sample stability was much improved compared to that for intact parathyrin measurement by RIA. The correlation studies showed a degree of correlation consistent with other comparisons similarly performed.

Introduction

The quantitation of parathyrin and its utility in discrimination of hypercalcaemic patients has been contentious since the capability of detecting the hormone was first established and controversy has continued to rage despite the development and refinement of antisera to parathyrin (1–3).

Part of the problem in the application of parathyrin assays to routine clinical practice lies in the very low (10^{-12} mol/l) concentrations of biologically active hormone in the blood but also in the heterogeneity of the circulating hormone.

In the past, antibodies used in C terminal parathyrin assays have been specific for amino-acid residues 53–84 of the parathyrin molecule. These assays will detect intact parathyrin, large parathyrin fragments containing the mid region and the C terminal, and small fragments consisting of the C terminal without the mid-region. Non-parathyrin proteins may interfere with binding in these assays causing falsely elevated values and secondly, biologically active components are short lived which can result in apparently low values if parathyrin secretion is episodic.

The low concentration and difficulties of isolating intact parathyrin from immunologically similar components have hampered development of assays. A major step forward was achieved by the introduction of pre-precipitation, partial purification and concentration steps which isolated intact parathyrin and other N-terminal fragments from the major biologi-

cally inert C-terminal fragments which made possible the quantitation of intact parathyrin within the normal range. There was however still a lack of precision and lack of sensitivity which did not allow the definition of a lower limit of normal such that suppressed parathyrin levels could be adequately defined (3).

The development of IRMA techniques has revolutionised other fields of endocrine hormone assay where there has been a need for improved detection limits and imprecision for the clinical application. It was predicted that a similar benefit would accrue by application of these techniques to the assay of intact parathyrin. A number of commercial kits have been developed in an attempt to achieve this. Here we describe an analytical assessment of two of the more popular IRMA kits for intact parathyrin against our current routine parathyrin assay.

Materials and Methods

Analytical evaluation

The analytical assessment consisted of comparing two IRMA kits (Kit A and B) for whole molecule parathyrin with an established RIA whole molecule parathyrin kit (Kit C) currently in routine use.

Kit A

Ntact® parathyrin IRMA kit product ca. 22800 (Incstar Corporation, Stillwater, Minnesota) was obtained from Immuno Diagnostic Systems (Usworth Hall, Washington, Tyne & Wear, or latterly direct from Incstar Ltd., Winnersh, Berkshire).

Kit B

Allegro™ Intact parathyrin immunoassay kit product no. 40-2170 (Nichols-Institute, San Juan Capistrano, California) was obtained from Biogenesis Ltd. (Yeomans Way, Bournemouth).

Both kits are of the immunoradiometric assay type, utilizing two different polyclonal antibodies purified by affinity chromatography. The first antibody specific for the mid range and C terminal portions (parathyrin 39–84) is immobilized onto plastic leads. The second antibody specific for the N terminal region (parathyrin 1–34) is labelled with ^{125}I. Intact parathyrin present in a sample is bound by both the immobilized and labelled antibodies to form a sandwich complex.

Following incubation, the bead is washed to remove unbound components and the radioactivity bound to the solid phase is measured in a gamma counter. The radioactivity of the bead bound complex is directly proportional to the amount of intact parathyrin in the sample.

Kit C

Human Ntact® parathyrin by RIA product no. 69065 (Incstar Corporation, Stillwater, Minnesota). This measures intact parathyrin utilising extraction followed by conventional radioimmunoassay technique. Extraction and concentration of intact parathyrin in plasma is achieved using specific absorption particles which remove only intact parathyrin and N terminal fragments. Following elution of parathyrin from these particles, assay by RIA is performed.

Following a period of familiarisation with the kits, evaluation was carried out as detailed adhering where appropriate to the ECCLS guidelines (4).

An established sample data base was already in existence within the laboratory, consisting of samples collected and stored under optimum conditions.

Statistical evaluation

The assay limit of detection was defined as the concentration corresponding to the 95% confidence limits of counts obtained for replicates of zero standards assayed in a single batch.

Regression and correlation studies were carried out using standard *Deming* regression equations. The straight line model correlation coefficient was used based on the *Deming* plots.

Results

Imprecision

Ten replicates of pooled human serum were analyzed in a single batch to assess within batch imprecision. Overall (between-batch) imprecision was assessed using the same serum pool assayed in duplicate in each of 11 assays. The results of within batch and overall imprecision are shown in table 1. For an equivalent number of assays, the Ntact RIA kit showed an overall CV of 14.2% at a mean concentration of 74 ng/l.

Assay limit of detection

Twenty replicate analyses of a zero standard were made and the limit of detection defined as given in the method section. The limit of detection was shown to 2.36 ng/l for the Ntact IRMA kit and 3.71 ng/l for the Allegro kit. The Ntact RIA kit has a limit of detection of 28 ng/l based on the same algorithm.

Dose response and parallelism

Using a range of dilutions of the International Reference Preparation for parathyrin (IRP 79/500) obtained from the National Institute for Biological Standards and Control, linearity was assessed over the concentration range 31.25 to 10000 ng/l (fig. 1). All dilutions of the IRP were prepared in phosphate buffered saline (pH 7.4) containing 300 g/l albumin.

Parallelism was investigated using 2 human plasma samples known to contain elevated concentrations of parathyrin. Varying dilutions of these were made in the kit zero standard. The concentrations obtained were corrected for the dilution factor and expressed as a percentage of the concentration measured in the undiluted sample (tab. 2).

Tab. 1. Imprecision study of parathyrin IRMA kits

	Incstar Ntact	Nichols Allegro
Within-batch imprecision using pooled serum		
$\bar{x}$ (ng/l)	37.18	43.22
SD	2.38	3.02
CV%	6.40	6.98
N	10	10
Overall imprecision using pooled serum		
$\bar{x}$ (ng/l)	38.84	44.19
SD	5.07	5.28
CV%	13.12	11.96
N	11	11

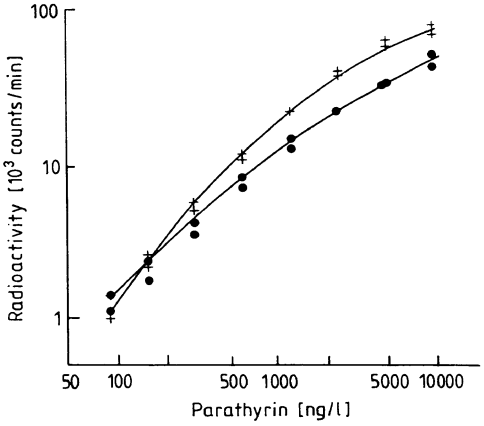

Fig. 1. Dose response study.
+ Incstar Ntact kit
● Nichols Allegro kit

Tab. 2. Parallelism study of parathyrin IRMA kits

Sample	Dilution factor	Measured parathyrin (ng/l)	Result corrected for dilution factor (%)
Incstar Ntact kit			
1	Undiluted	398	100
	1:2	160	80.4
	1:4	88	88.4
	1:8	41	82.4
2	Undiluted	363	100
	1:2	149	82.1
	1:4	83	91.5
Nichols Allegro kit			
1	Undiluted	372	100
	1:2	161	86.6
	1:4	77	82.8
	1:8	39	82.8
2	Undiluted	278	100
	1:2	124	89.2
	1:4	59	84.9
	1:8	34	97.8

Standard and quality control cross over study

Preliminary investigation had suggested that the results obtained with the IRMA kits were not directly comparable. To investigate the possible cause of the discrepancy, standards and quality control materials from each kit were assayed using the alternative kit reagents. The results are shown in table 3.

Tab. 3. Cross-over study of parathyrin IRMA kits using kit standards and quality control materials.

Kit standards

Assay of Incstar Ntact standard using Nichols Allegro kit		Assay of Nichols Allegro standards using Incstar Ntact kit	
Quoted concentration (ng/l)	Measured concentration (ng/l)	Quoted concentration (ng/l)	Measured concentration (ng/l)
0	<0	0	13.12
10	7.94	16	32.6
50	44.5	50	65.9
150	136.7	160	175.9
340	445.2	470	489.0
2000	1802	1650	1637

Quality control materials

QC Specimen	Incstar Ntact	Nichols Allegro	Manufacturer's quoted QC limits	
			Mean	Range
	Parathyrin concentration			
	ng/l	ng/l	ng/l	ng/l
1. Incstar QC1	83.9	54.0	66.8	51.6–82.1
2. Incstar QC2	707.1	417.0	579.0	462–696
3. Allegro QC1	56.9	38.8	53.8	35– 63
4. Allegro QC2	394.2	267.7	361.0	263–443

Accuracy

Accuracy was assessed by assaying known concentrations of parathyrin prepared from dilutions of IRP 79/500 in zero standard. The Incstar IRMA kit measured between 60% and 182% of the calculated dose at concentrations of 1000 and 62.5 ng/l respectively; whilst the Nichols kit measured between 42% and 80% of the calculated dose at the same concentrations.

Kit stability

Standard curves from IRMA kits were compared newly delivered, and two weeks past expiry date (fig. 2).

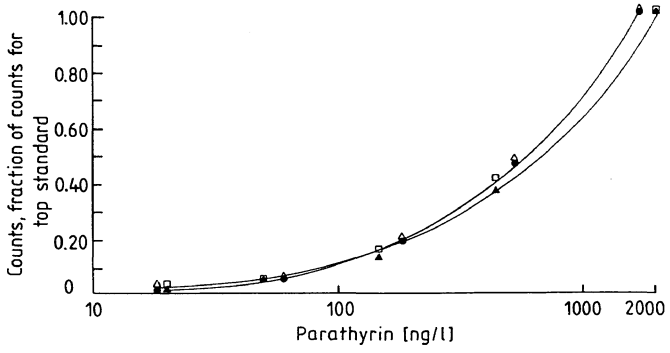

Fig. 2. Kit stability study
△ Allegro kit at delivery
● Allegro kit two weeks post expiry date
□ Ntact IRMA kit at delivery
▲ Ntact IRMA kit two weeks post expiry date

Correlation

Patient samples which had been stored under optimal conditions were analysed using the IRMA kits. All samples had previously undergone parathyrin assay using the Ntact RIA kit and covered a concentration range 28 – 270 ng/l by this technique.

For the possible combinations (Nichols vs Incstar IRMA, Incstar RIA vs Incstar IRMA and Incstar RIA vs Nichols), straight line correlation coefficients in excess of 0.9 were obtained for 88 data pairs.

Specimen requirements

Since parathyrin is a labile molecule, specimen collection and storage are of the utmost importance. Blood samples from 4 patients were collected into plain glass bottles for serum harvest and into bottles containing potassium-EDTA as preservative for plasma harvest. These samples underwent varying treatment with regard to separation and storage. All sample from an individual patient were analyzed in a single batch to obtain the results given in table 4. A difference in concentration from samples separated within 30 minutes at + 4 °C and stored at − 20 °C of greater than ($2 \times SD_{analytical}$) was taken as significant change.

Other factors

Laboratory staff involved in the evaluation were asked to record their subjective opinions of each kit with regard to packaging, instructions, ease of use etc. The cost per patient sample was also calculated.

Discussion

The contribution of parathyrin assay to the differential diagnosis of patients with hypercalcaemia has been contentious since its estimation was established. A number of factors appear to contribute to the various claims, amongst which are the parathyrin moiety used as a measure of circulating parathyrin status and the performance characteristics on the technique chosen to measure this.

Most commercial endeavour has been invested in improving the assay of whole molecule parathyrin. The two commercial kits evaluated here have been developed utilising IRMA technology (5, 6), and the manufacturers claim significant improvement in the analytical and clinical diagnostic utility in hypercalcaemic patients.

Minor improvements in overall imprecision were noted for both IRMA kits compared with the current radioimmunoassay at equivalent concentrations, which may reflect the avoidance of an extraction procedure.

The limit of detection for both IRMA kits showed approximately a 10 fold improvement over the conventional technique and should therefore permit identification of patients with suppressed levels of parathyrin. This improvement was not reflected by a greater improvement in assay precision but it is possible that had we assessed RIA precision at a parathyrin concentration closer to that used in the IRMA kit quality assurance material the difference might have been more marked.

Although standards provided in the kits only range from 0 – 2000 ng/l (Incstar Ntact) or 0 – 1650 ng/l (Nichols Allegro), the theoretical working range of the assays is approximately 5 times this concentration range. A standard curve prepared from IRP 79/500, showed acceptable linearity with no evidence of a high dose hook effect up to 10 000 ng/l.

Tab. 4. Assessment of specimen storage and stability

Conditions for collection and storage	Parathyrin concentration (ng/l)							
	Patient A		Patient B		Patient C		Patient D	
	Serum	Plasma	Serum	Plasma	Serum	Plasma	Serum	Plasma
(a) *Incstar Ntact kit*								
1. Separation within 30 min at +4 °C. Store −20 °C	26.2	33.0	109.1	–	31.4	48.5	100.3	124.3
2. Separation within 30 min at R. T. Store at R. T. for 24 h	11.2*	38.9	75.3*	140.8	16.4	54.8	69.3	157.3
3. Separation within 30 min R. T. Store −20 °C immediately	23.8	40.5	87.8	140.8	24.8	53.5	77.3	130.1
4. Separation within 30 min R. T. Freeze – thaw × 2	24.8	37.8	91.3	147.8	19.9	52.6	96.5	135.3
5. Separation at R. T. after 2 h at R. T.	32.1	37.2	84.8	146.3	21.0	46.0	95.2	138.8
6. Separation at R. T. after 24 h at R. T.	12.7	52.0	72.2	142.2	10.5	50.0	82.1	143.1
(b) *Nichols Allegro kit*								
1. Separation within 30 min at +4 °C. Store −20 °C	37.6	40.2	120.2	122.3	47.4	50.3	117.6	123.0
2. Separation within 30 min at R. T. Store at R. T. for 24 h	16.8*	32.8	84.2*	103.7	29.0*	47.8	88.6*	131.0
3. Separation within 30 min R. T. Store −20 °C immediately	38.1	39.6	126.6	112.6	44.0	45.2	95.4	121.8
4. Separation within 30 min R. t. Freeze – thaw × 2	35.9	37.3	111.0	118.4	35.2	43.0	121.2	116.5
5. Separation at R. T. after 2 h at R. T.	37.1	42.0	109.2	118.6	45.3	55.1	108.2	129.4
6. Separation at R. T. after 24 h at R. T.	23.1*	41.0	89.7*	104.2	29.3*	49.0	106.9	129.2

* indicates significant difference from row 1
R. T. = Room temperature

Patient samples when diluted showed recoveries as low as 80%. Neither kit seemed significantly better than the other. However with the theoretical working range of 0–10 000 ng/l, few clinical samples would require dilution if a wider range of standards were made available.

Problems experienced in the initial phase of this study led us to question the comparison of results obtained by the IRMA techniques. Whilst a significant correlation coefficient was obtained, a few patient samples appeared grossly discrepant. Crossover of kit standards and quality control materials showed a similar discrepancy. Wheter this represents differing assignments for standards against the IRP, or varying antisera avidity is not clear.

It is interesting that a similar discrepancy was shown in the accuracy study utilising the IRP. Neither kit quotes the basis of their standardisation and whilst the Allegro kit underestimated the concentration across the whole standard range, the Ntact kit appeared to overestimate at lower concentrations and underestimate at higher concentrations.

Both IRMA kits were shown to be acceptable for use outwith their quoted expiry dates which is advantageous if regular useage is not envisaged.

Deming analysis showed good correlation between the concentrations measured by the IRMA kits when compared with the existing whole molecule assay.

One major point highlighted in this technical evaluation was that of improved sample stability over that seen in the RIA assay routinely. The existing requirement has been for plasma samples collected, separated and frozen within 30 minutes. After this period a marked decline in the concentration of intact parathyrin measured had been noted (7).

Utilising the IRMA kits however, a less rigid requirement was demonstrated. Standing for up to 2 hours at room temperature appeared to have little effect on the measured dose, providing that samples were then stored frozen. Plasma samples seemed less sensitive to storage conditions than serum samples. This is in contrast to the findings of *Newman & Ashby* (8).

Subjective impressions of both IRMA kits were favourable compared to the RIA method which involves an extraction procedure. In terms of hands on time however, all three assays were comparable. The IRMA kits were less expensive per patient sample than the RIA kit although the assay turn round time is longer for the IRMA kit. In a practical situation however this fact is irrelevant.

As neither of the IRMA kits under assessment showed significant technical superiority over the other, the decision of which kit to use rests soley on the performance in the clinical setting. The clinical assessment is proceeding at present and will be reported on when completed.

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